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Gene transfer into the central nervous system using *Herpes Simplex Virus-1* vectors

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Manipulation of gene expression in developing or in mature central nervous systems (CNS) holds a promise for the resolution of many compelling neurobiological questions, including the feasibility of gene therapy to treat diseases of the brain. In this context, a number of viral vectors have been used in recent years to introduce and express genes into the CNS. This article discusses a gene transfer system based on the Herpes Simplex Virus-1 (HSV-1). We describe here the use of non-replicating, non-toxic HSV-1 vector, 8117/43, in a series of studies carried in our joint program. This vector proves further the utility of HSV-1 as a delivery vehicle to a number of distinct sites within the CNS.

key words: *Herpes Simplex Virus-1*, substantia nigra, hippocampus, *β*-galactosidase, gene therapy

INTRODUCTION

Interest in introducing genes into the CNS has focused attention on a number of viral vectors. Several aspects of the natural biology of HSV-1 make it an attractive vector for delivering and expressing foreign genes within the nervous system. First and foremost is the fact that HSV-1 is a neurotropic virus possessing a sophisticated genetic program allowing it to spend most of its life cycle within the nervous system. HSV-1 enters neurons at the periphery and travels via axonal transport to the cell bodies of the sensory ganglia where it becomes latent [18,20]. HSV-1 has the capacity to travel transsynaptically far from its original site of infection. In humans, most infections are sub-clinical, and by age 20 approximately 90% of the population has been exposed to HSV-1. During latency the virus's 152 kb double-stranded DNA genome persists as circular episomes within the nuclei of neurons. It is the ability of the virus to naturally co-exist within the nervous system without causing disease (or provoking the immune system) that suggests its suitability as a vector. Some of the key advantages of HSV-1 vectors are that: 1) they establish a life-long latent infection within peripheral and CNS neurons; 2) latent HSV-1 genomes exist as multiple episomal copies per neuron and integration is not known to occur [16]; and 3) non-replicating HSV-1 recombinants can establish a latent infection efficiently [17]. This last point is perhaps the most important in that it permits the construction of safe, attenuated vectors for human gene therapy. In addition, there are other biological properties of HSV-1 which enhance its suitability as a vector from a practical standpoint. The virus is easy to manipulate *in vitro*, so that recombinants containing foreign genes can be constructed rapidly and

its genome can accept large inserts of DNA, making the construction of vectors (which express multiple therapeutic genes) feasible. This potential has understandably generated a great deal of interest in exploiting HSV-1 as a vector [10], and to date a number of recombinants have been generated expressing reporters such as β-galactosidase [1,5,6,9], as well as biologically relevant peptides such as glucouronidase [21], tyrosine hydroxylase [7] and nerve growth factor (NGF) [8,14]. These vectors include both recombinant HSV-1 and a derivative termed "amplicons". Here we will be discussing only the former.

Engineering of HSV-1

Early viral constructs expressed their respective markers transiently at high levels, however, the expression declined rapidly with time. While for some therapeutic applications transient expression of a peptide within target neurons may be sufficient, for most uses long-term expression from the latent infection is desirable. Stable expression of genes from the context of the latent viral genome has proven the most difficult problem to solve in the development of HSV-1 vectors. Since latent infections of HSV-1 are characterized by the absence of viral transcription (with the exception of the latency associated transcript, LAT [19]), the LAT promoter would be the ideal candidate for the expression of foreign genes during latency. However, recombinant viruses containing the genes for nerve growth factor and β-galactosidase driven by the LAT promoter express β-galactosidase and NGF RNA at high levels initially, but not during the latent infection [14]. A weaker promoter downstream of the LAT promoter (LAP2) has also been shown to be insufficient for long-term

expression. A number of cellular promoters, including those for neuronal housekeeping genes, have been evaluated for their long-term ability to express genes in the nervous system within the context of the HSV-1 genome. These constructs expressed reporter genes at high levels during the acute infection (for a period of 2–10 days p.i.) but the levels of expression dropped off dramatically following the latent infection [3]. The Moloney Murine Leukemia Virus (MoMuLV) LTR has been demonstrated, however, to afford long-term expression of β-galactosidase [4,6] in the context of the LAT promoter [13]. This combination of the LAT-core promoter and the MoMuLV LTR allows extended expression of transgenes at high levels within the sensory neurons of the peripheral nervous system [14], but only minimal levels of sustained expression within the CNS [4]. Recently, the native region of the HSV-1 genome, which may allow sustained expression, has been further localized to an element downstream of LAT, termed LTE for long-term expression [2,12]. In addition, there is also evidence that a region upstream of the LAT core promoter may play a role in enhancing long-term expression properties [15]. Clearly the regulation of this region is complex, but enough details are now available that reasonable levels of "long-term" expression in the CNS can be achieved for at least several weeks, which is sufficient for a variety of research applications.

In the following section we describe the use of the non-replicating HSV-1 vector, 8117/43. This vector is ICP4 minus [i.e., it has the major viral transactivator gene (Infected Cell Protein 4; ICP4) deleted] and is completely non-replicating and must be propagated on a helper cell line that provides the ICP4 gene *in trans*. As shown in Figure 1, this vector con-
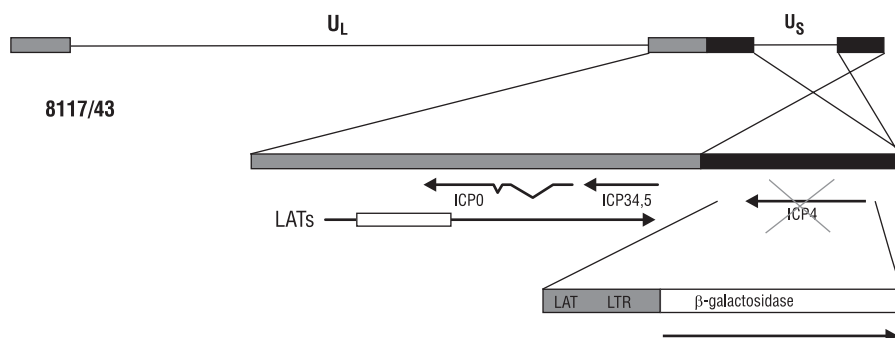

Figure 1. Diagram of the non-replicating HSV-1 recombinant 8117/43. The HSV-1 genome (top) is double stranded DNA 150 kb in length and is comprised of 4 components: the unique long (UL) and unique short (US) segments (indicated by the thin lines), and the long repeat (RL) and short repeats (indicated by the stippled boxes, and black boxes, respectively). The region comprising the junction of one of the long repeats and one of the short repeats is expanded below the diagram to illustrate the site into which the E.coli *LacZ* has been inserted in place of the ICP4 gene of the virus, driven by the LAT/MoMuLV LTR promoter (see text). KD6, and ICP4-minus virus made by transfecting HSV-1 strain KOS DNA with engineered plasmid DNA in which most of the coding sequence for ICP4 had been deleted, served as a control virus in some of our experiments.

tains the *E. coli* LacZ gene driven by the LAT/Mo-MuLv LTR promoter [6]. This vector demonstrates the utility of HSV-1 as a delivery vehicle to a number of distinct sites within the CNS.

METHODS

Animal groups

Adult Male Fischer 344 rats (n = 27) Harlan Sprague-Dawley Inc., NIA colony) with average body weight 250 g were used. The animals were housed in the Laboratory Animal Facilities in the School of Medicine and Biomedical Sciences at the State University of New York at Buffalo. They were kept in stainless steel wire cages in a room with 12 hours light/dark cycle, and were fed a standard laboratory diet (Prolab R-M-H 1000, Agway Inc., Syracuse, NY) and water *ad libitum*. Before surgery, animals were randomly assigned to one of three groups:

I. Experimental group, inoculated with HSV-1 (8117/43) [4 μl, 3 x 10^6 plaque forming units (PFU) per injection site] (n = 14).

II. Control group, injected with 4 μl of 5% glucose per injection site (n = 9).

III. Control group inoculated with HSV-1 (KD6) lacking β-galactosidase reporter gene (4 μl, 3 x 10^6 PFU per injection site) (n = 4).

All rats were inspected visually each day and weighed each week to monitor health. All procedures were approved by the Institutional Animal Care and Use Committee guidelines (IACUC) of the State University of New York at Buffalo.

Stereotaxic inoculation

Each animal was weighed then deeply anesthetized with a mixture of ketamine (100 mg/ml), xylazine (20 mg/ml), and ace-promazine (10 mg/ml) in 0.9% NaCl (0.2 ml/100 g body weight, i.p.). The rat was situated in the stereotaxic apparatus, and the skull was revealed through a 1cm incision. Bregma location was recorded and the surgical coordinates were established for the injection sites. A 1 mm hole for each injection site was drilled in the skull using a dental drill. Stereotaxic intracerebral injections were performed using 30 G stainless steel needle of the 10 μl Hamilton syringe held by the micromanipulator on the stereotaxic apparatus. Rats in all groups were injected with 4 μl/injection site of the proper solutions. The solutions were injected at 1 μl/min, and the injection was followed by a 4 min waiting period before the needle was removed from the brain.

Injection sites

Each rat was stereotaxically injected bilaterally in the rostral hippocampus and substantia nigra. The following coordinates were used to inject into the hippocampus: Anterior-posterior (AP): −3.60 mm, medial-lateral (ML): ±2.40 mm and dorsal-ventral (DV): −3.40 mm. Substantia nigra coordinates were: AP: −5.60 mm, ML: ±1.50 mm., and DV: −8.20 mm. After the last injection, the incision was closed with biodegradable sutures and the rats were housed one per cage, and their health was closely monitored until full recovery.

Survival duration following surgery

All groups were divided into three subgroups according to the time of sacrifice post injection:

Short term (ST): sacrificed at 5 days post-injection
Mid term (MT): sacrificed at 14 days post-injection
Long term (LT): sacrificed at 26 days post-injection

Tissue preparation and histochemical procedures for the detection of β-galactosidase

At various times post injection, each rat was deeply anesthetized (0.8 cc, i.p.) as determined by the absence of responses to afferent stimuli. Rats were perfused transcardially by 100 mM PBS (pH 7.3) solution followed immediately by ice-cold fresh 4% paraformaldehyde fixative in 0.1 M phosphate-buffer (pH 7.3). Rats were decapitated, brains were removed and placed in the 4% paraformaldehyde fixative solution. Brains were cut into 1 mm coronal sections using stainless steel brain mold. During cutting, brain tissue was moistened by paraformaldehyde to prevent drying. These 1mm serial sections were transferred into individual wells of 12-well dish and post fixed with 2% formaldehyde, 0.2% glutaraldehyde, 0.1% sodium deoxycholate and 0.02% NP-40 in 100 ml of 100 mM PBS for 1 hour at 4°C. Following post fixation, tissue was washed twice with PBS followed by one wash with 3% DMSO/PBS. The X-gal staining solution consisted of 0.87g NaCl, 100 mM HEPES (pH 7.5) 2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide. This solution was freshly prepared and kept at 4°C. When ready to use, the solution was warmed to 36°C and 5 ml of 2 % X-gal [5-bromo-4-chloro-3-indolyl β-D-galactopyranoside] in dimethyl formamide were added. Tissues were stained with X-gal for 7 hours at 31°C in the dark. Subsequently, the slices were washed several times in PBS and placed in 30% sucrose in PBS. All solutions were prepared fresh and

their pH was adjusted above 7.2 in order to prevent the detection of endogenous (acidic) β-galactosidase. Digital pictures were taken and the tissues were further cut into 50 μm sections using a freezing microtome. Brain sections were mounted onto gelatin subbed slides, counter stained with Eosin Y, then subjected to routine dehydration through ethanol. Finally, sections were passed through xylene and cover slipped with paramount.

DNA and RNA extraction from dissected samples of rat CNS

Homogenization of tissue: Frozen tissue samples were homogenized in a total of 4 ml of Trizol (Life Technologies, Gaithersburg, MD). The solution was collected in a microcentrifuge tube and incubated for 5 min at room temperature. Following incubation, 200 μl of chloroform was added to each sample and samples were vortexed for 15 sec. Samples were then incubated for an additional 5 min at room temperature, and microfuged at 9,000 x g (10,500 rpm in a Brinkman microfuge) for 15 min at 4°C to ensure separation of the aqueous and organic phases.

Extraction of the RNA fraction: The aqueous phase was removed and transferred to a clean microcentrifuge tube and 500 μl of isopropanol was added. The tube was shaken for 15 sec, incubated for 5 min at room temperature and then microfuged at 9,000 x g for 10 min. The samples were washed with 75% ethanol (made with DEPC-treated water) and centrifuged for 5 min at 4°C at 8,500 rpm. The RNA pellet was air dried, resuspended in 200 μl of DEPC-treated water, and incubated for 5–10 min at 55°C to resuspend the RNA. 100 μl of the 200 μl sample was DNase-treated with RNase-free DNase (Stratagene), using 20–40 U in 20 mM Tris, pH 7.4; 10 mM MgCl$_2$ for 30 min at 37°C. DEPC water was added to bring the total volume to 200 μl, the sample extracted with phenol/chloroform/isoamyl (mix phenol 1:1 to chloroform/isoamyl) (chloroform/ isoamyl 24:1) extract in (1:1) ratio. The aqueous layer was taken and extracted again with chloroform/isoamyl (1:1). The aqueous layer was taken and transferred to a new centrifuge tube and the RNA precipitated by adding 1/50 volume of 5 M NaCl and 2.5 volume of 100% ethanol. The samples were microfuged at 14,000 rpm in the Brinkman microcentrifuge for 15 min at 4°C. The tubes were rinsed with 1 ml of 75% ethanol and microfuged for an additional 5 min at room temperature and briefly air-dried. The RNA was resuspended in 50 μl DEPC-treated water.

Preparation of cDNA: 0.5 μg of RNA (usually in 5––10 μl of the sample from above) was reverse-transcribed into cDNA. The reaction volume of 20 μl contained: 5–10 μl of sample RNA, 4 μl of 5x RT buffer (Life Technologies), 1 μl (10 pmol) random hexamer primers (Pharmacia), 1 μl 12.5 μM cold dNTPs (Pharmacia), 1 μl MoMuLV RT (Life Technologies), 0.5 μl RNasin (Promega). The samples were mixed gently for 1–2 sec and then incubated at 37°C for 1 hour. The reactions were stopped by heating at 100°C for 10 min, then quenched on ice and microfuged for 1–2 sec.

PCR reactions: The PCR reactions were set up as follows: 5 μl of 10X PCR buffer (1.5 mM Mg), 1 μl of dNTP (12.5 mMol), 1 μl of primer 1, 1 μl primer 2,1 μl of the cDNA sample, 0.5 μl of Taq (Perkin-Elmer), 40.5 μl of water for a 50 μl total reaction. The PCR conditions for detection of the β-gal transcripts were 94°C, 55°C, 72°C for 3 min (1X) and 94°C, 55°C, 72°C for 1 min (30X). The primer set for β-gal detection: Primer 1: 5'ATG GCT GAC TGG GGG GCA TAT3'; Primer 2: 5'GCA CAG TGA AAT CGT TAA TAT3'.

RESULTS

Gene therapy, a procedure which employs the use of viral vectors to deliver therapeutic genes into neuronal cells, has exciting clinical implications as a tool for treating human neurodegenerative diseases. In this context, two candidate neural systems, the basal forebrain-hippocampal projection implicated in Alzheimer's and the nigrostriatal pathway implicated in Parkinson's, appear to be model systems to test gene therapy procedures. Using these two neural pathways, we investigated the expression of β-galactosidase reporter gene following the injection of non-replicating HSV-1.

Injection into hippocampus

Inoculation of the 8117/43 virus into the rostral hippocampus resulted in strong expression of recombinant viral β-galactosidase activity with a great number of cells labeled around injection site (Fig. 2A). At 5 days post injection the stained region extended at least 3 mm in the anterolateral direction and 3–5 mm in mediolateral direction (Fig. 2A,B). Scattered cells of the medial septum/diagonal band were positively stained following injection into this region (Fig. 2B). This may be a result of retrograde transport of the virus. Figure 2C shows labeling of hippocampal neurons.

The initial (i.e., 5 days) strong expression in the hippocampus was significantly reduced 15 days post-inoculation and little or no expression was detected 26 days post-inoculation. (Fig. 2A). The expression of β-galactosidase activity in basal forebrain was tran-
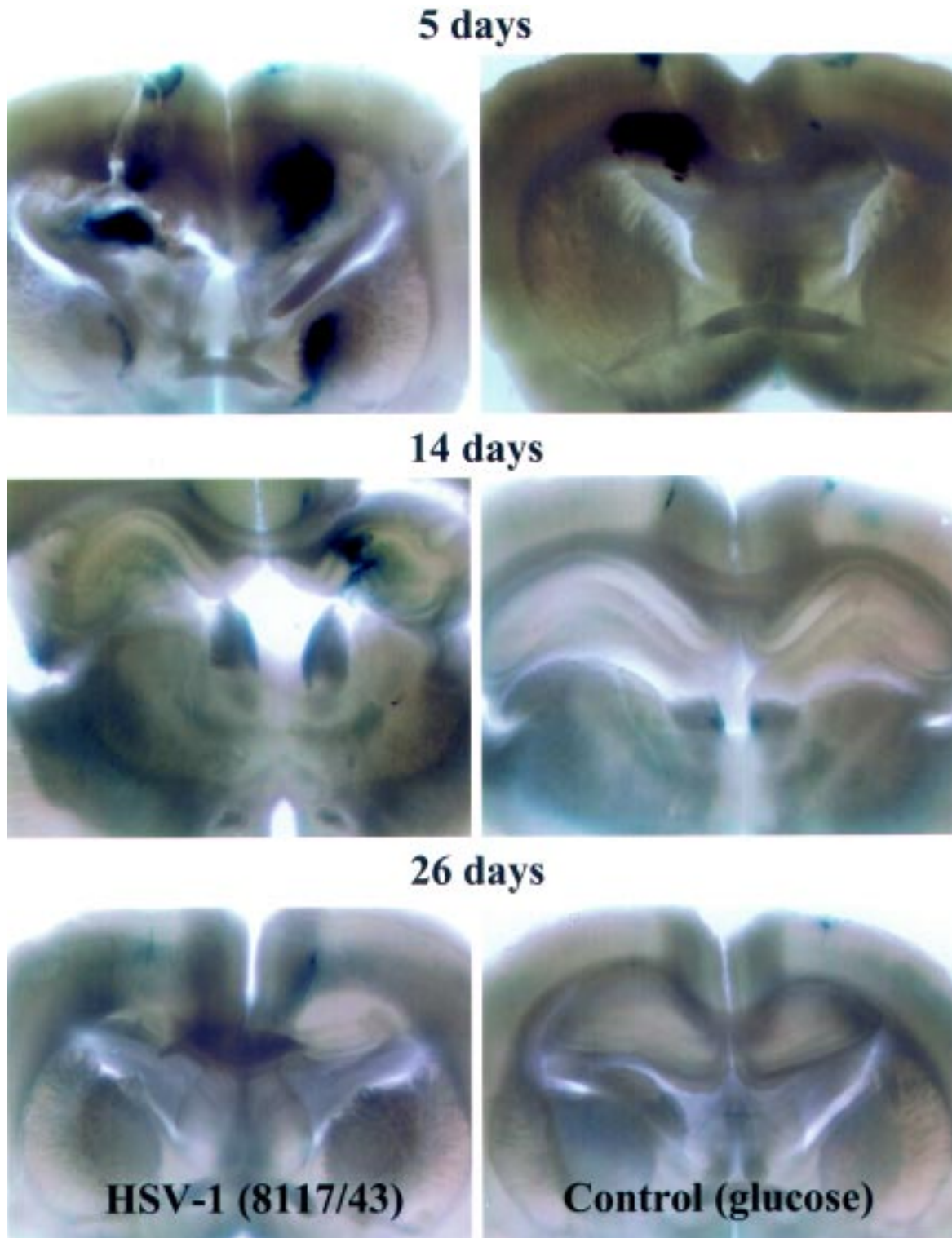
5 days

14 days

26 days

HSV-1 (8117/43)   Control (glucose)

Figure 2. Histochemical detection of recombinant β-galactosiadse activity in the hippocampal, striatal, and basal forebrain regions. Rats received a bilateral inoculation of HSV-1 8117/43 or injection of 5% glucose (control) into rostral hippocampus and substantia nigra. **A**) 1 mm sections photographed immediately following incubation with X-gal staining solution for 7 hours. X-gal staining (dark blue) detects the expression of exogenous β-galactosidase following the injection of 4 μl of β-galactosidase DNA. HSV-1 (8117/43) injection produces widespread expression of the reporter gene β-galactosidase in the hippocampus at 5 days post injection. The expression was decreased significantly at 14 days post injection until it disappeared at 26 days. No exogenous β-galactosidase activities were detected in the hippocampal regions in the control sections. Minor blue background was evident in some 1 mm sections especially at the needle tracks and choroids plexus regions, however, this blue background was not cellular (see figures 2C and 3C).
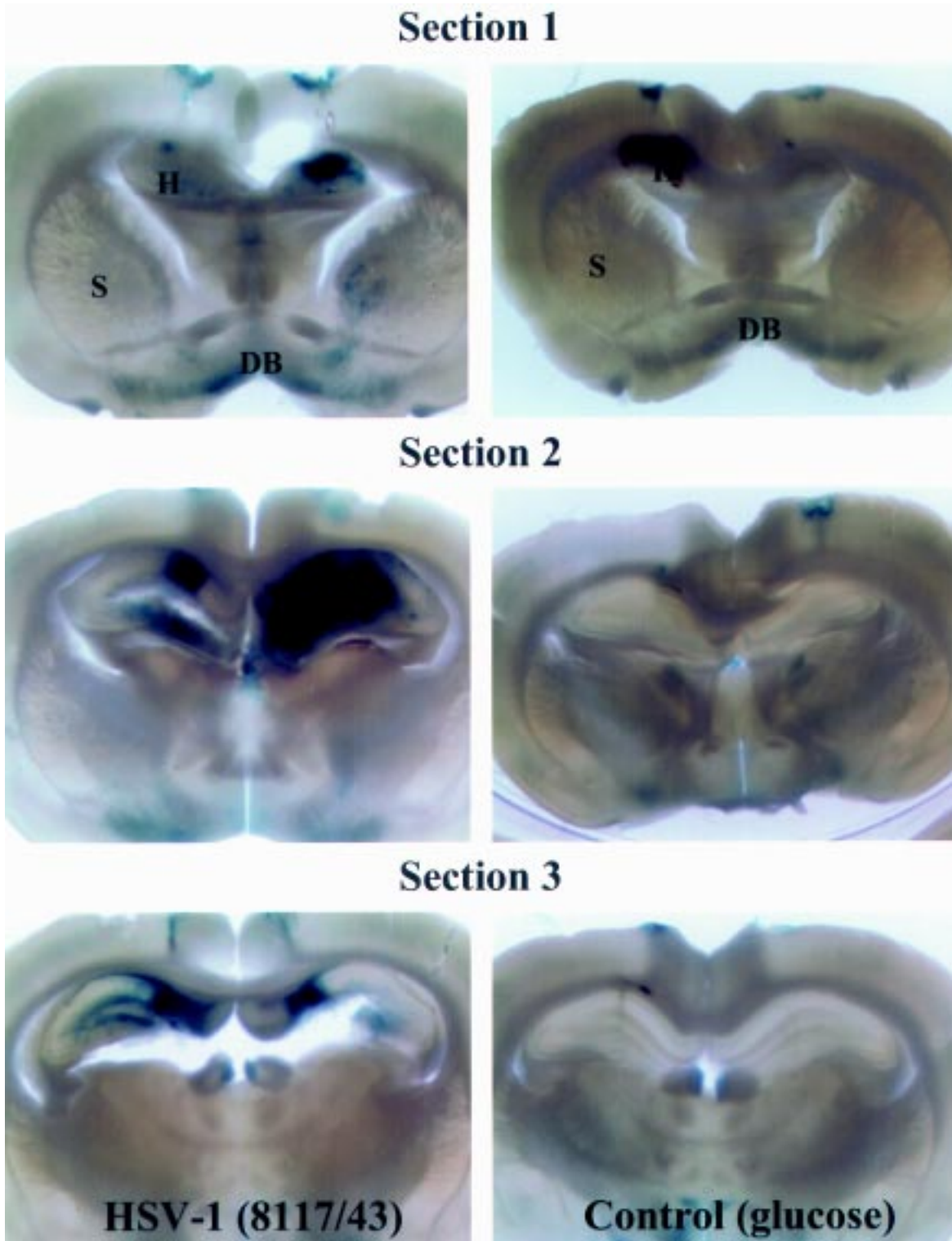
Fig. 2B. Three consecutive 1 mm coronal sections throughout the rat hippocampus. HSV-1 8117/43 vector or 5% glucose (control) was injected bilaterally into the hippocampal region. The activities of recombinant β-galactosidase were revealed five days post injection by the chemical reaction with X-gal (dark blue). Sections show β-galactosidase expression in the hippocampus following the use of HSV-1 8117/43 as a vector. No β-galactosidase expression in the control sections was detected. H — hippocampus, S — substantia nigra, DB — nucleus of the diagonal band.
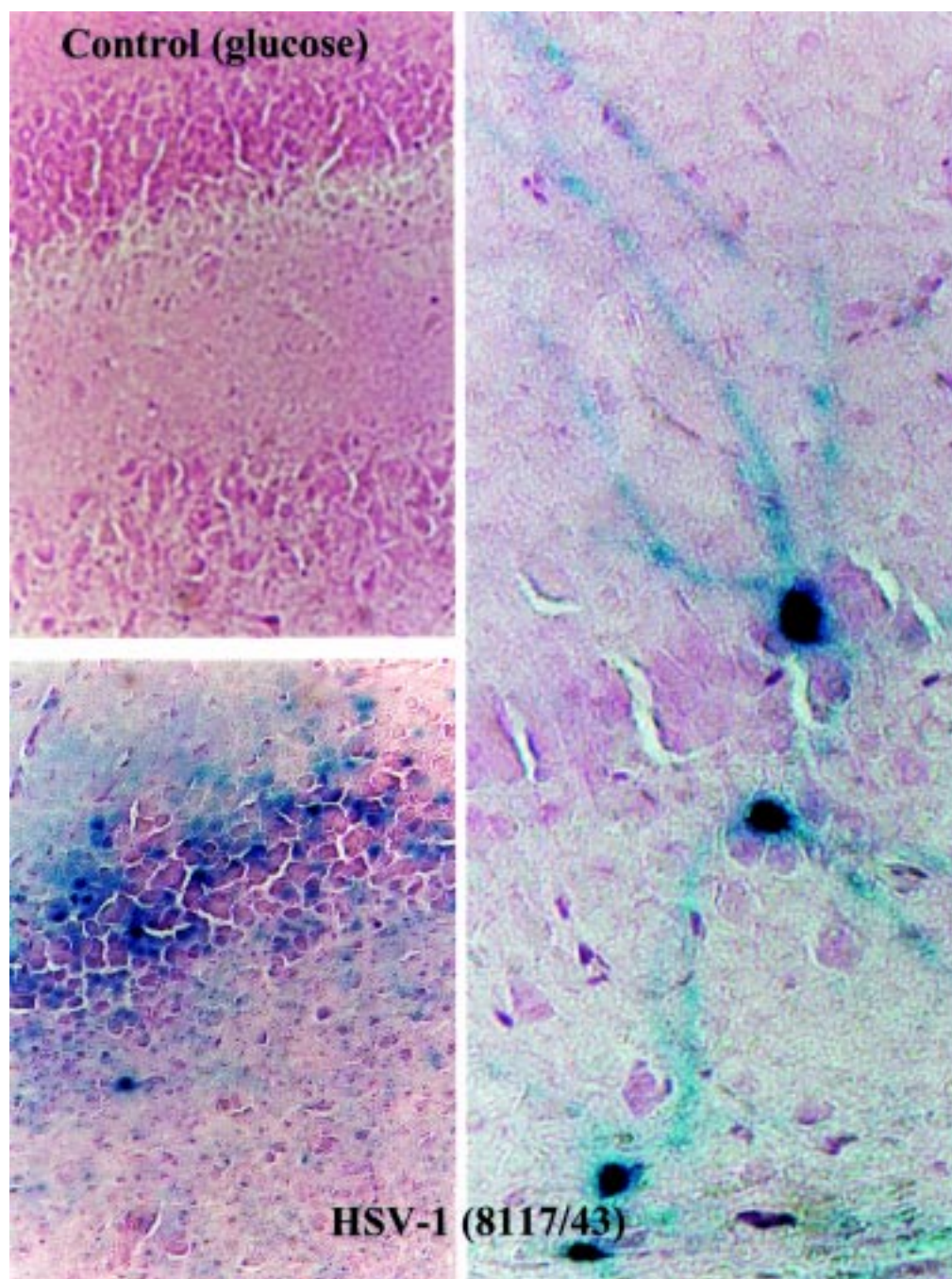
Fig. 2C. Expression of β-galactosidase in the hippocampus inoculated with HSV-1 8117/43 (10x). The neurons expressed β-galactosidase activity in soma and dendrites (Figure at the right, 20x). Five days post injection (50 μm sections).

sient (similar to hippocampal expression). In control brains injected with 5% glucose solution no staining was detected at any time after injection.

Injection into substantia nigra

Initially (i.e., 5 days), inoculation into the substantia nigra resulted in a relatively weak X-gal staining, reflecting limited number of neurons expressing β-ga-

lactosidase activity (Fig. 3A). An X-gal positive neuron in the zona compacta of the substantia nigra is shown on Figure 3B. Interestingly, with time the expression of β-galactosidase activity increased, and 26 days after the inoculation a great number of cells showed strong β-galactosidase activity (Fig. 3A,C). No staining was observed at any time in control brains injected with glucose (Fig. 3A,C). Also, in

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5 days

14 days

26 days

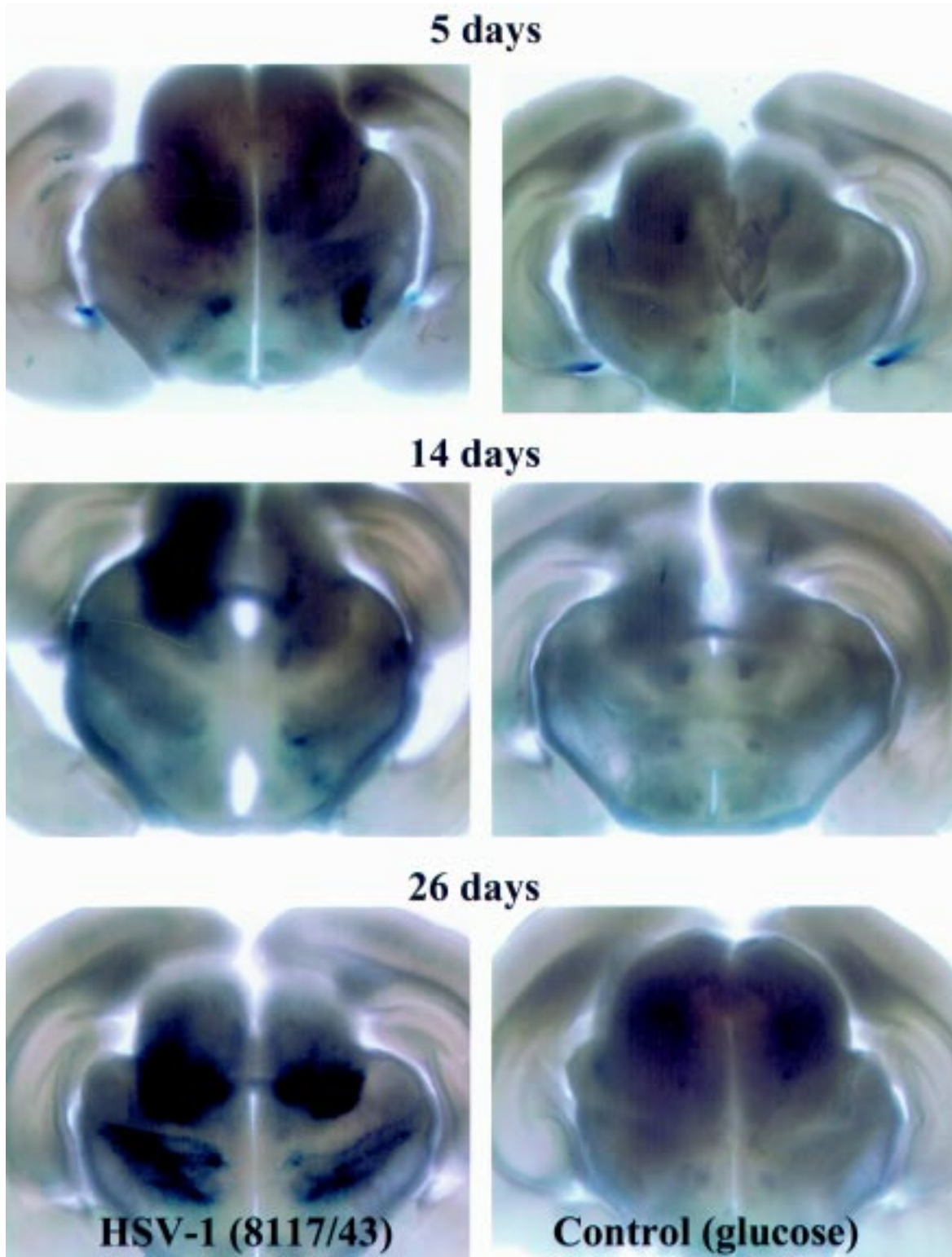**HSV-1 (8117/43)**   **Control (glucose)**

Figure 3. Figure represents the results of the stereotaxic injection of HSV-1 8117/43, control HSV-1 KD6, or 5% glucose into the substantia nigra region.
A) Expression of β-galactosidase in substantia nigra at several time points post injection (1 mm brain sections) (10x).
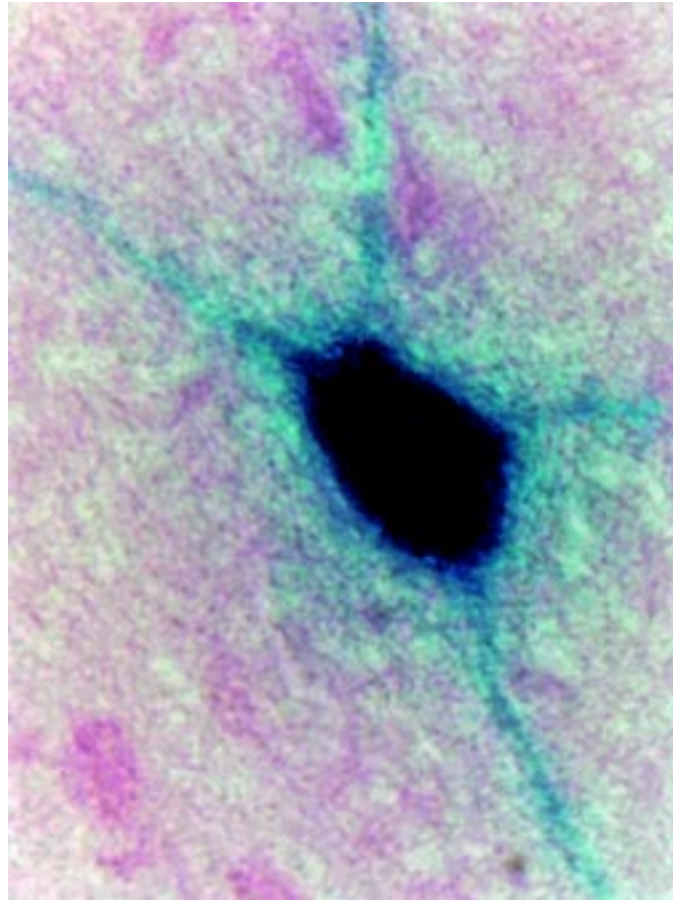
Fig. 3B. Expression of β-galactosidase by HSV-1 8117/43 in substantia nigra neurons (40x). Five days post injection (50 μm sections).

brains inoculated with control HSV-1 (KD6) no β-galactosidase activity was detected (Fig. 3D).

Spread of the virus from the substantia nigra was examined by determining whether specific staining was present in the striatum after virus injection. A transient X-gal staining in the striatum was observed at 5 days and only trace amounts at 14 days following the virus injection (Fig. 2A,B). Under higher magnification the staining was localized to striatal neurons, apparently due to retrograde transport of the virus by the striatonigral pathway (data not shown).

To ascertain that the HSV-1 8117/43 remained in the substantia nigra region for an extended period of time, the presence of viral DNA was examined using PCR 26 days after the inoculation. Viral DNA remained present for almost 1 month after the inoculation (Fig. 4).

Unlike earlier studies which utilized HSV-1 amplicon that may be contaminated with "helper virus" [7], no pathological changes (i.e. gliosis, neuronal degeneration) were detected in brain regions inoculated with HSV-1 8117/43.

DISCUSSION AND CONCLUSIONS

1. Our studies have shown that the non-replicating HSV-1 8117/43 construct, in which the Lac Z gene is driven by combined LAT and MoMuLV LTR promoters, is capable of expressing the reporter gene in a number of different neurons within the CNS. The high relative levels of the reporter gene expression were observed in neuronal cell bodies directly within the inoculated area but also in afferent projections. The retrograde spread of the virus from substantia nigra to striatum via striatonigral projection and from the hippocampus to basal
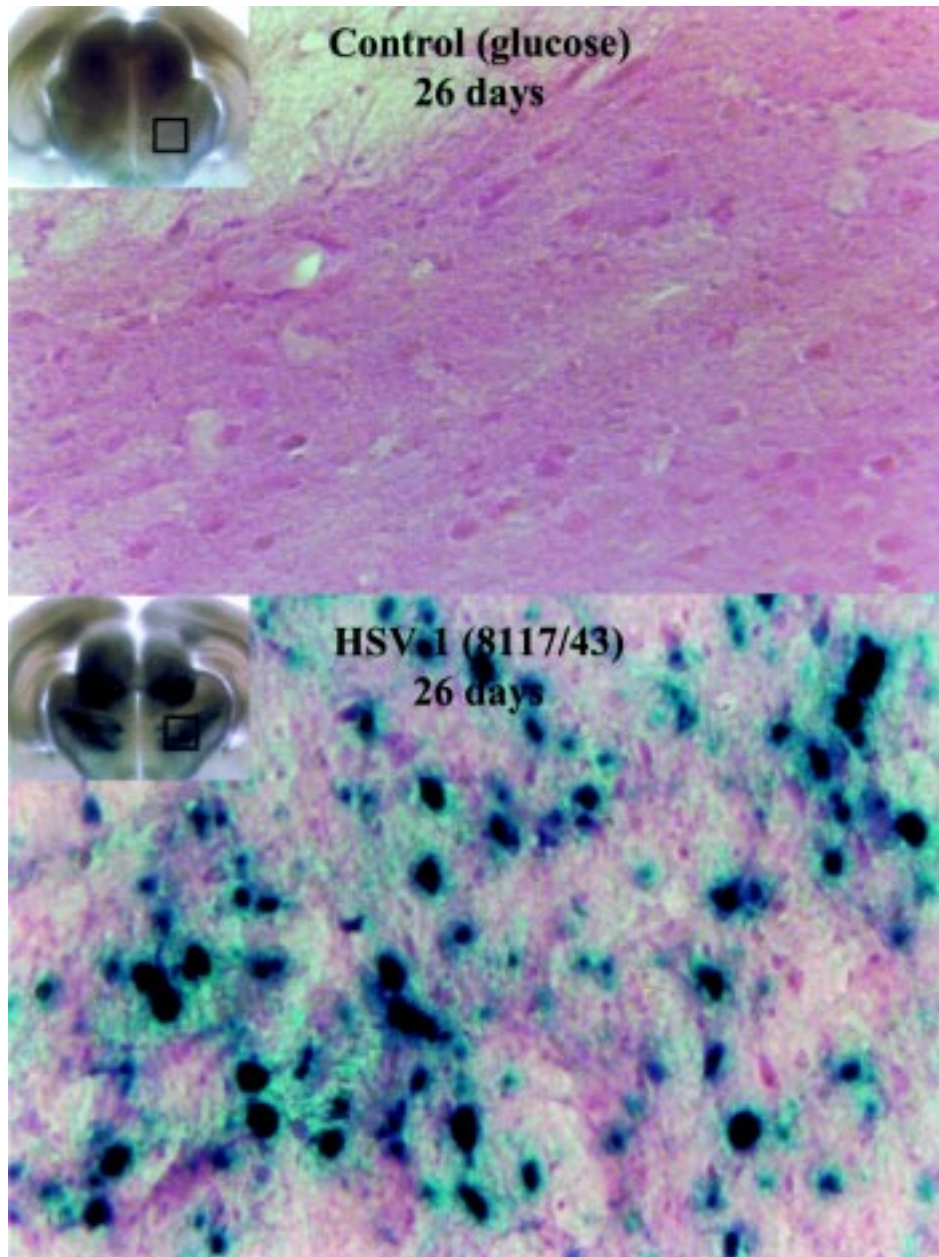
Fig. 3C. Substantia nigra 26 days post injection (50 μm sections) (20x).

forebrain through the hippocampo-septal pathway, demonstrates the feasibility of using the 8117/43 virus to express genes in areas distal from the injection site, thus preserving local microanatomy.

2. The propensity of wild-type HSV-1 for retrograde and anterograde transport and for trans-neuronal transfer has been used to trace neuronal pathways in the brain [11]. However, we found no evidence for the trans-synaptic viral spread. This is consistent with the earlier finding that after the deletion of the immediate early gene ICP4, the ability of the virus to replicate or reactivate from the latent state was completely abolished [4,6].

3. Although the length of expression (several weeks) of HSV-1 8117/43 may not be sufficient to offer an immediate therapeutic promise, this length of expression and the number of cells expressing the reporter gene are both significant for more acute basic applications. These may include in vivo intracerebral gene transfer to investigate underlying mechanisms and potential corrective therapies for neurodegenerative diseases, especially those involving the nigrostriatal pathway.
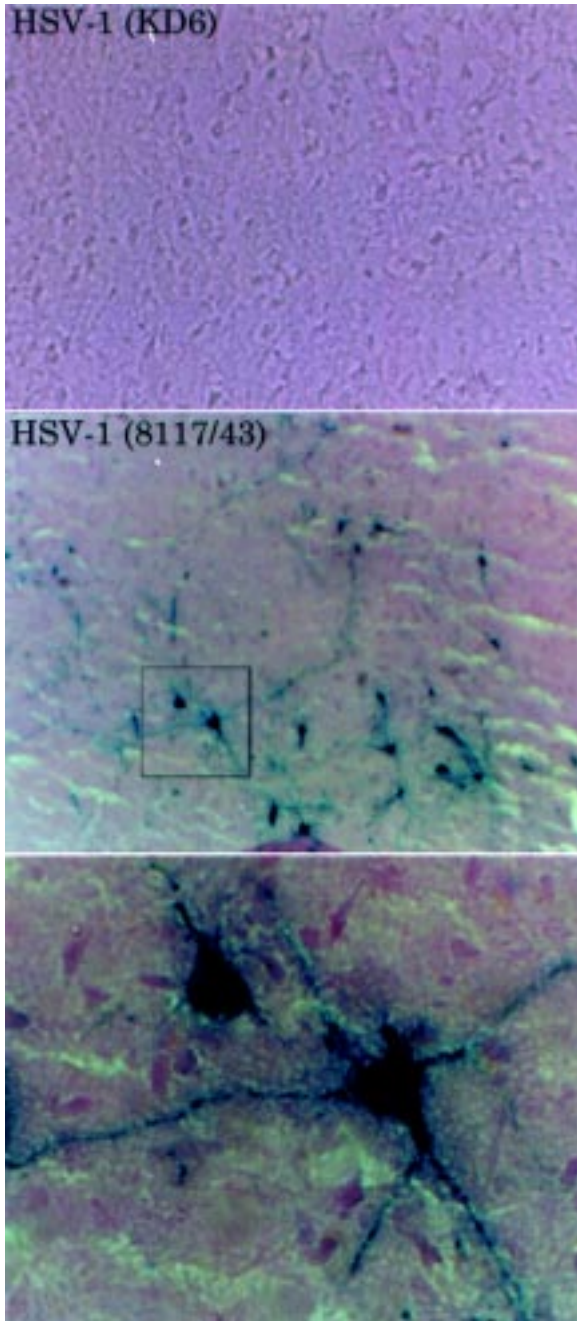
HSV-1 (KD6)

HSV-1 (8117/43)

Fig. 3D. Substantia nigra was inoculated with 8117/43 HSV-1 or control KD6 HSV-1. Histochemical detection of β-galactosidase expression in substantia nigra 6 days after inoculation (50 μm sections; top and middle panel – 10x; bottom panel – 40x).
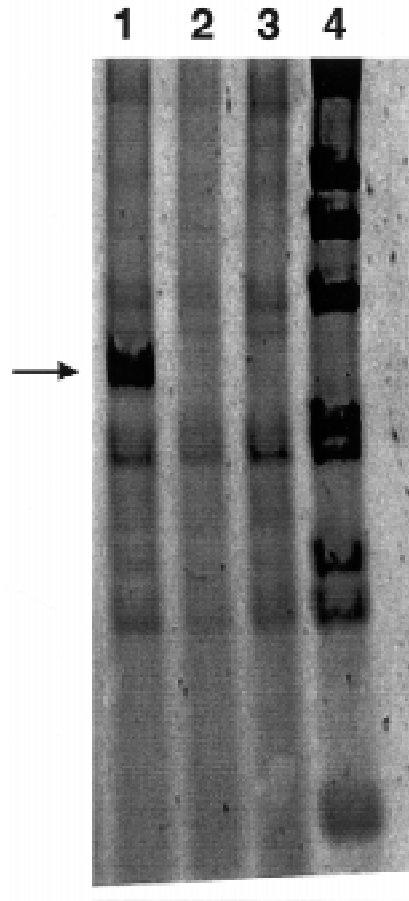

Figure 4. RT-PCR analysis of dissected regions of rat CNS for β-galactosidase RNA. 26 days following inoculation of rat CNS with the HSV-1 vector 8117/43, rats were sacrificed and 1–2 mm regions from the substantia nigra dissected and subjected to RT-PCR analysis as described in Materials and Methods. Primers specific for β-galactosidase were used and the resulting 293 bp PCR product is indicated by the arrow. Rats inoculated with either 8117/43, glucose (control), or β-galactosidase-expressing plasmid DNA mixed with polyethyleneimine (PEI) are compared. Lane 1 — 8117/43; Lane 2 — Glucose; Lane 3 — PEI, Lane 4 — size standards.

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